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Effect of hypo– and hypersaline stress conditions on physiological, metabolic, and immune responses in the oyster *Crassostrea corteziensis* (Bivalvia: Ostreidae)

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ABSTRACT

Salinity in the oceans is changing due to climate change and global warming. Intense rainfalls and freshwater runoff decrease salinity along the coastal areas. In contrast, intense drought seasons and river damming have certainly increased salinity in lagoons and estuaries. Few studies have focused on aspects of the biology and culture of oyster *Crassostrea corteziensis*, but until now, physiological and immunological responses in this species have not been assessed under acute hypo– and hypersaline stress conditions. Oysters obtained from a local farm were acclimated for three weeks in laboratory conditions. To avoid closure of oyster valves during salinity induced–stress conditions, a notch was done on each organism shell not only to facilitate oyster tissue exposure to rearing water but also for sampling hemolymph. Oysters (N = 180) were abruptly exposed to three salinity treatments: (HO) hypo–, (C) control, and (HP) hypersaline stress conditions (10, 35, and 50 PSU, respectively). Four oysters per treatment were sampled at 1, 2, 3, 6, 12, 24, and 48 h after exposure. Hemolymph osmolality, water content and total protein concentration in tissues, metabolic and immune responses were assessed for each organism. Oyster survival was not different among treatments and was maintained above 96% at the end of the experimental trial. Hemolymph osmolality reached the value of rearing water at 6 and 48 h of exposure to HP and HO stress conditions, where oysters exposed to salinity increase showed less resilience than those to decrease. Higher glucose levels in plasma and lower ones of hemocyanin were assessed in the oysters exposed to HP compared to HO conditions, suggesting more stressful conditions or susceptibility of oysters during salinity increase. Total hemocyte (THC), hyalinocyte (HC), and granulocyte (GC) counts decreased in oysters exposed to HP condition, while total and differential hemocyte counts were similar among oysters exposed to HO and control conditions. Despite hemocyte phagocytosis was not different among treatments, viability decreased in those exposed to HP condition. Contrastingly, superoxide anion (SOA) production (oxidative capacity) increased in oysters exposed to both induced salinity–stress conditions, which suggest susceptibility increase in oysters, particularly during salinity increase. The results show that HP condition is particularly stressful for *C. corteziensis*. In turn, this condition could increase both their vulnerability to other environmental stressors, such as temperature and/or acidification or susceptibility to opportunistic pathogenic microorganisms that cause the most common oyster diseases.

1. Introduction

Bivalve mollusk production is the second largest in aquaculture industry with an estimated production of 17.5 M t in 2018 [1]. In Mexico, total oyster production in 2019 was estimated in 55 355 t, where the oyster *Crassostrea corteziensis* (Hertlein, 1951) was the third most harvested species with a production of 1851 t in 2019 [2]. Despite their importance as one of the most reared species, lack of knowledge still remains in several aspects of their biology, mainly in regard to their immune system. For instance, probiotics have been tested on superoxide dismutase (SOD) activity in *C. corteziensis* spats exposed to pathogenic bacteria [3]. When compared with a continuous–dripping feeding system, a once daily batch increased hemocyte oxidative capacity (superoxide anion [SOA] production) without observing differences in total and differential hemocyte counts between both feeding systems [4]. Additionally, incorporating arachidonic acid (20:4n–6) –as lipid

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emulsion in the diet of adult *C. corteziensis*– enhanced total and differential hemocyte counts and SOA production [5]. More recently, metabolic, immune responses and HSP70 expression in adult *C. corteziensis* hemocytes showed that thermic and thermic–mechanical stress conditions were more stressful than mechanical disruption. Additionally, females were observed to be more susceptible than males when exposed to these stressors during rearing [6]. Furthermore, seasonal variation in *C. corteziensis* immune response has demonstrated an increase in total and differential hemocyte counts (THC and DHC), and SOA production during winter. These results coincide with an increase of toxic dinoflagellates, particularly when some species of the genus *Prorocentrum* are abundant [7], which were confirmed where *C. corteziensis* was exposed to different cellular densities of *Prorocentrum koreanum* at different times of exposure. The results on immune response suggest that this species is capable of resisting short periods of intoxication, while THC and phagocytosis increased in particular [8]. Salinity –combined with temperature effect– has been only assessed in relation to feeding activity to evaluate scope for growth (SFG) of *C. corteziensis* exposed to these important environmental parameters, where filtration and clearance rates, feed assimilation efficiency and SFG, decreased as salinity increased [9].

Bivalve mollusks are osmoconformers, that is, instead of maintaining hemolymph osmolality independently of external salinity changes, it changes concomitantly with environmental osmotic pressure changes when the organisms are induced to saline stress condition [10]. Salinity stress–induced is known to possibly impair different physiological (growth, feeding activity, heart rate, and oxygen consumption), metabolic (protein use as source of amino acids as organic osmolytes for adjusting cell volume, and glycogen mobilization), and immune response (humoral and hemocyte–mediated) functions in different bivalve mollusks [for a review see Ref. [11], which eventually may cause significant bivalve mollusk mortalities and economic losses [12–14]. Salinity stress–induced affects both humoral responses not only by increasing defensive peptides [15] but also cellular response. In this situation, from different bivalve species an increase in hemocyte mortality [16–18] and immune system suppression were observed in the mussels *Mytillus edulis* [19] and *Mytilus cosruscos* [20], as well as in the clams *Chamelea gallina* [21], *Ruditapes philippinarum* [22–24], *Mercenaria mercenaria* [25], and *Paphia malabarica*[18] exposed to hyposaline (HO) stress condition. In contrast, both an increase in the immune response in the clams *Ruditapes philippinarum* and *Ruditapes decussatus* [12] and mussel *Mytilus galloprovincialis* [26], as well as immune suppression in the clams *Ruditapes philippinarum* [23] and *Chamelea gallina* [21], oysters *Ostrea edulis* [27] and *Crassostrea gigas* [16] were observed when they were exposed to the hypersaline (HP) stress condition. Finally, salinity stress–induced also increased susceptibility to certain pathogens in disease development, as observed in the *O. edulis* [27] and clams *R. philipinarum* [22] and *M. mercenaria* [25].

Ocean salinity is changing worldwide [28] due to climate change and global warming. This scenario has profound implications not only for ocean circulation and climate but also for both terrestrial and particularly the aquatic life it supports [29]. Intensive rainfalls and fresh water runoff decrease salinity in coastal areas, while intense droughts and river damming increase salinity in lagoons and estuaries. Thus, determining how salinity changes affect species physiology is important to understand their resilience capacity, allowing us to reduce economic losses and maintain their production to guarantee food security. Therefore, the aim of this research is to evaluate the physiological, metabolic, and immunological response of *C. corteziensis* oysters exposed to acute hypo– and hypersaline stress conditions.

2. Material and methods

2.1. Oysters and experimental conditions

Two hundred *C. corteziensis* oysters with 65–95 mm shell length were sampled from a local oyster farm located at Ceuta Bay, Sinaloa, Mexico. The oysters were acclimated for three weeks in aerated filtered seawater at 35 PSU and fed with microalgae *Chaetoceros mulleri* at a ratio of 5% of oyster tissue per dry weight daily. Once oysters were acclimated, a notch was done on the shells of each one of them, close to the adductor muscle not only to facilitate hemolymph extraction but also assure the proper exposure of oyster tissues to rearing water at hyposaline (HO) and hypersaline (HP) stress conditions [30].

The experimental trial was performed indoors with 60–L plastic tanks, and stock density was maintained at 20 oysters/tank for each treatment in triplicate. Rearing water was permanently aerated (dissolved oxygen >6 mg mL^{-1}) and maintained at 25 ± 1 °C by controlled room temperature. Oysters were abruptly transferred to the experimental conditions where two treatments and one control group were handled: HO (10 PSU), control (C) (35 PSU), and HP (50 PSU) conditions. Experimental water was previously prepared, maintained at the same temperature and oxygen levels, desired salinity using tap water for 10 UPS, and dissolved unprocessed marine salt (Salinera Punta Arenas, BCS, MX) for 50 UPS. Four oysters were sampled on each treatment at 1, 2, 3, 6, 12, 24, and 48 h after starting the experimental trial.

2.2. Hemolymph and tissues sampling

The maximum possible hemolymph volume samples were taken from each oyster by puncturing the adductor muscle vein using a 5–mL plastic hypodermic syringe. Each hemolymph sample was divided in five subsamples to measure osmotic pressure, biochemical composition (total protein and glucose content), hemocyanin, total and differential hemocyte counts, phagocytosis, super anion production and hemocyte viability. Then, each oyster was opened, dissected to obtain a sample of mantle, muscle, and gills, and samples were preserved at – 80 °C.

2.3. Tissue analysis

All tissue samples were thawed and weighed with an analytical balance. Later, the samples were lyophilized for 48 h, weighed to measure dry weight and assess water content. Total protein in tissues (mantle, muscle, and gills) was quantified according to the methodology described by Bradford [31]. Absorbance was determined at 595 nm using a spectrophotometer (Imark, Bio–Rad, U.S.A). Protein concentration was calculated using a standard curve from dilutions of bovine serum albumin (BSA) (A–2153, Sigma–Aldrich, St. Louis, MO, U.S.A) with the following concentrations: 5, 2.5, 1.25, 0.625, 0.3125, and 0.15625 mg/mL.

2.4. Biochemical plasma analysis

Osmolality in plasma was determined immediately after hemolymph was sampled, using a micro freezing osmometer (μOmette 5004, Precision Systems Inc., U.S.A.). All remaining hemolymph samples were centrifuged at 1300×*g* at 4 °C for 10 min to obtain plasma.

Total protein content in plasma samples was assessed by Bradford methodology [31]. Absorbance was determined at 595 nm using a spectrophotometer (Imark, Bio–Rad, U.S.A). Protein concentration was assessed as described above for protein content in oyster tissues.

One plasma sample was diluted with 3.5% NaCl in a 1:10 ratio; subsequently, absorbance was determined at 335 nm using a spectrophotometer (Jenway 7305, UK). To calculate hemocyanin concentration as a function of absorbance, an extinction coefficient (E1%) of 2.83 was applied [32].

Glucose was determined by the method glucose oxidase (GOP) – phenol aminophenazone (PAP) using a commercial kit (Randox) as described in a previous work [6].

2.5. Total and differential hemocyte counts

Total (THC) and differential (DHC) hemocyte counts were individually analyzed with an anti aggregant solution [33] by microscopy in hemolymph samples of each oyster as in previous works [5,6]. Cells were distinguished by their granule size and content. The results are expressed as total hemocytes, hyalinocytes, and granulocytes per milliliter of hemolymph.

2.6. Hemocyte viability

Functional hemocyte characteristics were analyzed once hemolymph osmolality reached that of rearing water (isosmotic point); since it occurred at a different time in the oysters exposed to both salinity induced–stress conditions, sampling and analyzing the immune response in both oyster groups had to be performed at the same time (48 h).

Hemocyte viability was individually analyzed using microscopy and trypan blue solution (T–8154, Sigma–Aldrich, St. Louis, MO, U.S.A) as in a previous work [6]. Stained dead hemocytes [34] are expressed as the percentage of total hemocytes counted per hemolymph aliquot.

2.7. Phagocytosis

Phagocytosis was analyzed using fluorescent microscopy and Fluoresbrite microspheres (Polysciences, Inc. Warrington, PA, U.S.A.) as previously described [6]. Phagocytosis is expressed as the percentage of hemocytes that engulfed two or more microspheres in relation to total hemocytes counted per hemolymph aliquot.

2.8. Superoxide anion

Superoxide anion (SOA) production [35] was analyzed immediately after hemolymph sampling as described in previous works [4,6]. Hemocytes were stimulated with laminarin (Sigma–Aldrich, U.S.A. Cat. No. L9634, 0.2 mg/mL final concentration) diluted in Hank's balanced salt solution (Sigma–Aldrich, H-8264). The results are reported as the ratio of stimulated/basal SOA activity [arbitrary units (AU)] of hemocyte concentration in the aliquot used for the analysis.

2.9. Statistics

Data obtained from the experiment were analyzed by two–way analysis of variance (ANOVA) using time of exposure (1, 2, 3, 6, 12, 24, and 48 h) and salinity (10, 35, and 50 PSU) as independent variables. The functional hemocyte characteristics (*i.e.* viability, phagocytosis, and SOA production) obtained from the organisms exposed only for 48 h were analyzed by one–way ANOVA using salinity (10, 35, and 50 PSU) as independent variable. When a significant effect was observed, means were compared with a post hoc Tukey's test to assess significant ($P < 0.05$) differences. In the data analyzed by two–way ANOVA, global means (separated only for the significant factor) were compared when a significant main effect was obtained for any of the two factors. Percentage results were transformed to arcsine before analyses [36], but only untransformed means are provided. Data are reported as mean ± standard error and were analyzed with Statistica v. 6.0 (Statsoft, Inc., Tulsa, OK, U.S.A).

3. Results

3.1. Osmolality in hemolymph

Significant differences were observed ($P < 0.01$) in hemolymph osmolality of oysters exposed to HO and HP stress conditions for 48 h (Fig. 1). In those exposed to HO stress condition (10 PSU), hemolymph reached the isosmotic point with rearing seawater after 48 h of exposure

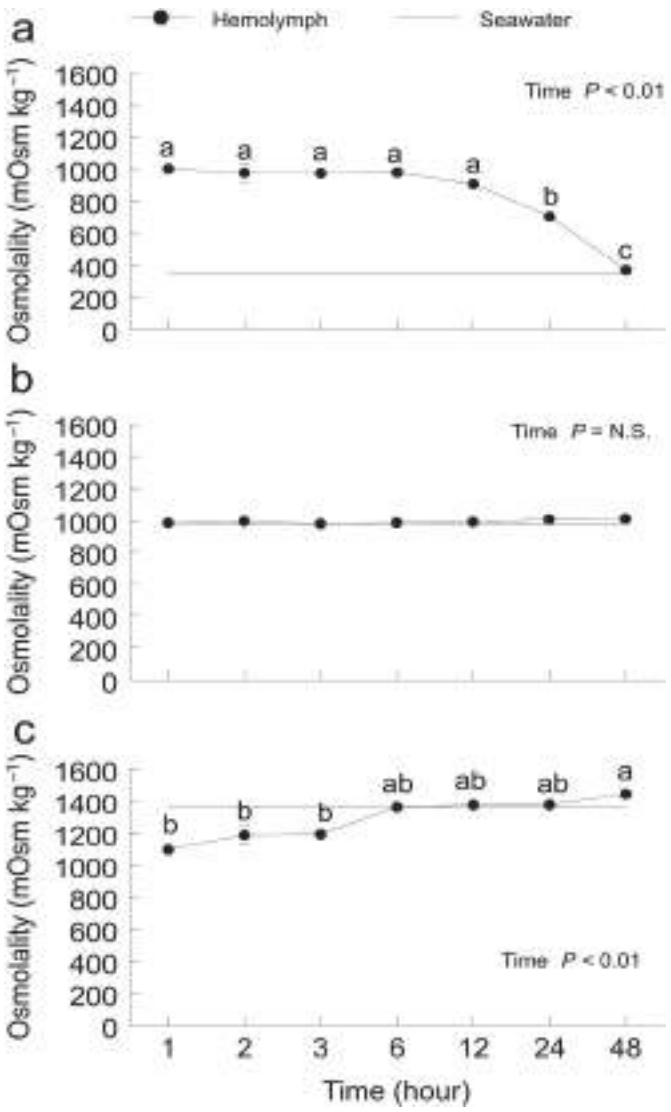

Fig. 1. Osmolality (mOsm kg^{-1}) in rearing water (continuous line) and hemolymph of oysters exposed from 1 to 48 h to acute **(a)** (HO) hypo– (351 mOsm kg^{-1}), **(b)** (C) control (982 mOsm kg^{-1}), and **(c)** (HP) hypersaline (1365 mOsm kg^{-1}) stress conditions (10, 35, and 50 PSU, respectively). Values are means ± standard error (SE) and were analyzed by one–way analysis of variance (ANOVA) and Tukey's test to assess significant differences. Means with different letters indicate statistically significant differences ($P < 0.05$).

with osmolality average of 371 ± 3.53 mOsm kg^{-1} (Fig. 1a). In contrast, the isosmotic point in hemolymph of oysters exposed to HP stress condition (50 PSU) was reached after 6 h of exposure with osmolality average of 1364 ± 1.19 mOsm kg^{-1} (Fig. 1c). As expected, the osmolality of oysters maintained at 35 PSU (control) did not change during the 48 h of exposure (Fig. 1b).

3.2. Water content in tissues

Significant differences ($P < 0.01$) were found in water content in the gills in relation to exposure time but not in regard to salinity and interaction of both salinity and time (Fig. 2a). Water content in oyster gills increased with exposure time from 79.9 ± 0.9% to 82.7 ± 1.1% from 1 to 48 h of exposure.

In the adductor muscle, water content changed ($P < 0.01$) in relation to salinity exposure, with higher values in oysters exposed to HO stress condition (77.9 ± 0.6%). While the lowest value (75.6 ± 0.6%) was obtained in those exposed to HP stress condition; an intermediate value

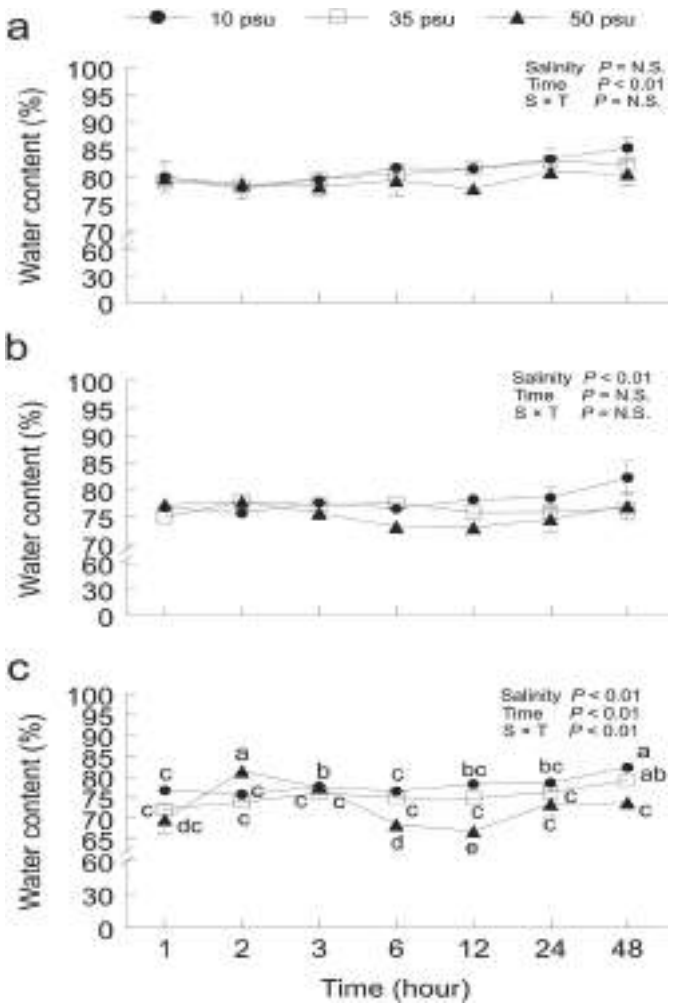

Fig. 2. Water content (%) in **(a)** gills, **(b)** adductor muscle, and **(c)** mantle in oysters exposed from 1 to 48 h to (HO) hypo–, (C) control, and (HP) hyper–osmotic stress conditions (10, 35, 50 PSU, respectively). Values are means ± standard error (SE) and were analyzed by two–way analysis of variance (ANOVA) and Tukey's test to assess significant differences. Means with different letters indicate statistically significant differences ($P < 0.05$).

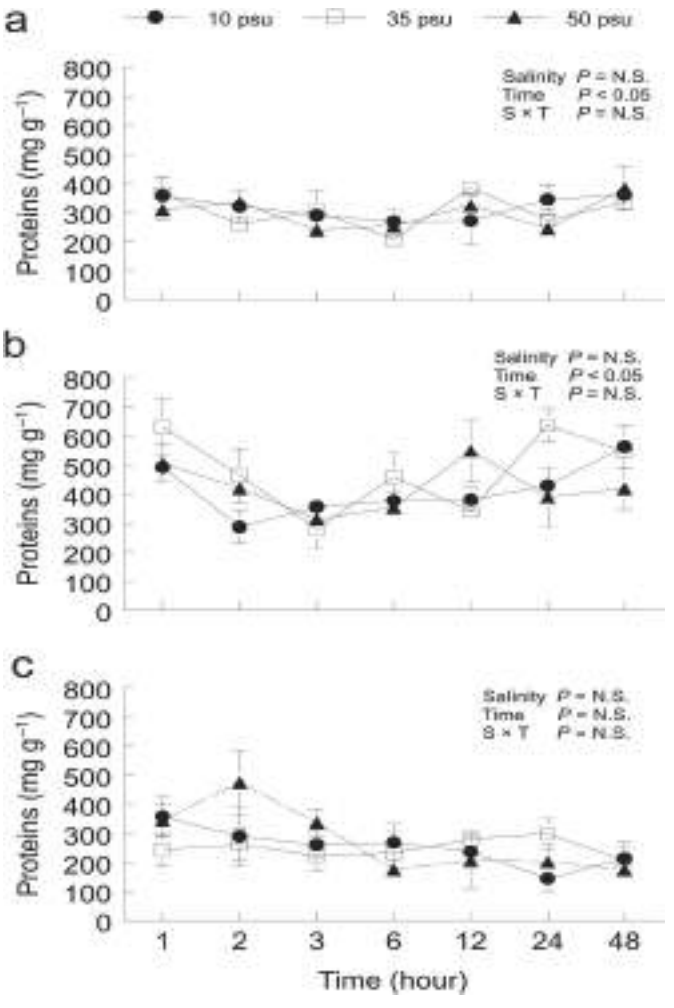

Fig. 3. Protein concentration (mg g^{-1}) in **(a)** gills, **(b)** adductor muscle, and **(c)** mantle of oysters exposed to (HO) hypo–, (C) control, and (HP) hyper–osmotic stress conditions. Values are means ± standard error (SE). See Fig. 2 for statistical analyses.

(76.5 ± 0.3%) was recorded in those maintained in C conditions (Fig. 2b). No significant differences were observed in water content of the adductor muscle in regard to exposure time or an interaction between both factors (salinity and exposure time).

In the mantle, water content (Fig. 2c) changed in regard to salinity exposure ($P < 0.01$), time of exposure ($P < 0.01$), and interaction between both factors ($P < 0.01$). Water content was higher (75.9 ± 0.8%) in oysters exposed to HO stress conditions, compared with those exposed to HP stress condition (72.4 ± 1.2%) with a middle value in those maintained in C condition (75.3 ± 0.6%). In regard to exposure time, water content increased from 72.1 ± 1.6 to 78.1 ± 1.4% from 1 to 48 h. A significant interaction was observed between salinity and exposure time with higher values in the mantle of those exposed 2 h to HP stress conditions (81.3 ± 0.8%) and a lower value (66.8 ± 2.0%) in those exposed 12 h.

3.3. Total protein in tissues

Total protein concentration in the gills changed ($P < 0.05$) in relation to exposure time, but no significant effect occurred with salinity change or interaction between both factors (Fig. 3a). Proteins in gills decreased with exposure time from 1 to 6 h (347–243 mg g^{-1} wt weight), then increased at 48 h even to higher values (360 mg g^{-1} wt weight) compared to those observed in the beginning of the experimental trial.

Similar results were obtained in the adductor muscle where total proteins changed in regard to exposure time, but no significant effect occurred with salinity changes or interaction between both factors (Fig. 3b). Proteins in the adductor muscle decreased with exposure time from 1 to 3 h (536–317 mg g^{-1} wt weight), then increased at 48 h to similar values (506 mg g^{-1} wt weight) observed at the beginning of the experimental trial.

Total protein content did not change in the mantle in relation to salinity changes, exposure time or an interaction between both factors (Fig. 3c).

3.4. Total proteins, hemocyanin, and glucose in plasma

No significant differences were found in total protein concentration in oyster plasma in regard to salinity changes, exposure time or interaction between both factors (Fig. 4a).

Significant differences were found in hemocyanin content among salinities ($P < 0.01$). The average hemocyanin content was higher in organisms exposed to HO stress condition (0.41 ± 0.07 mg mL^{-1}), followed by C (0.32 ± 0.04 mg mL^{-1}) and HP stress conditions (0.23 ± 0.02 mg mL^{-1}) (Fig. 4b).

Glucose content in oyster plasma changed in regard to salinity ($P <$

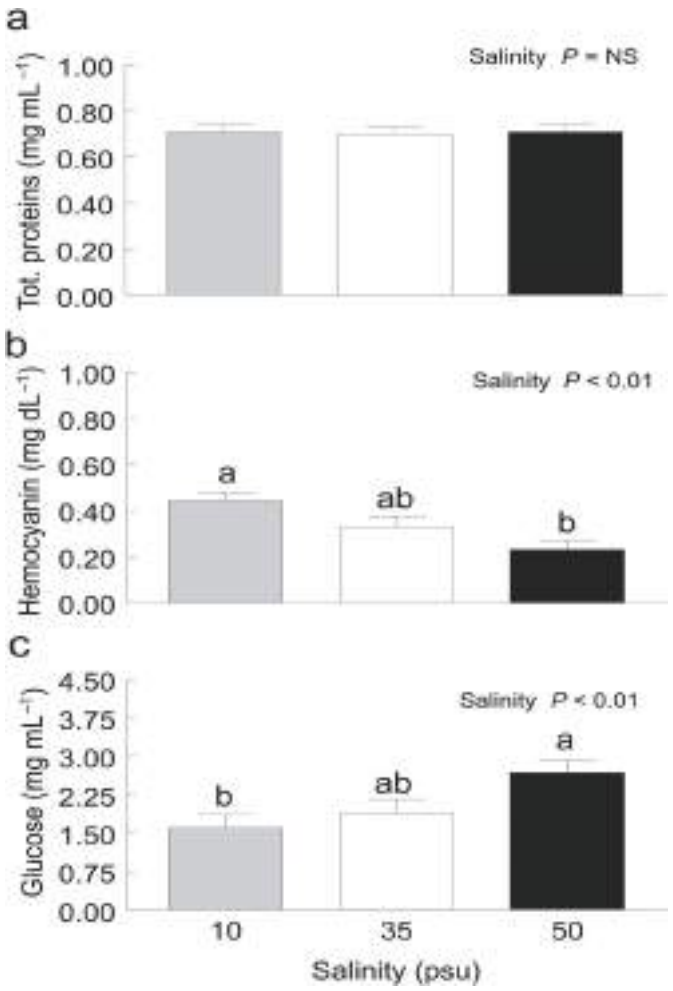

Fig. 4. (a) Total proteins (mg mL^{-1}), **(b)** hemocyanin (mg dL^{-1}), and **(c)** glucose (mg mL^{-1}), in plasma of oysters exposed from 1 to 48 h to (HO) hypo–, (C) control, and (HP) hyper–osmotic stress conditions (10, 35, and 50 PSU, respectively). Values are global means ± standard error (SE). See Fig. 2 for statistical analyses.

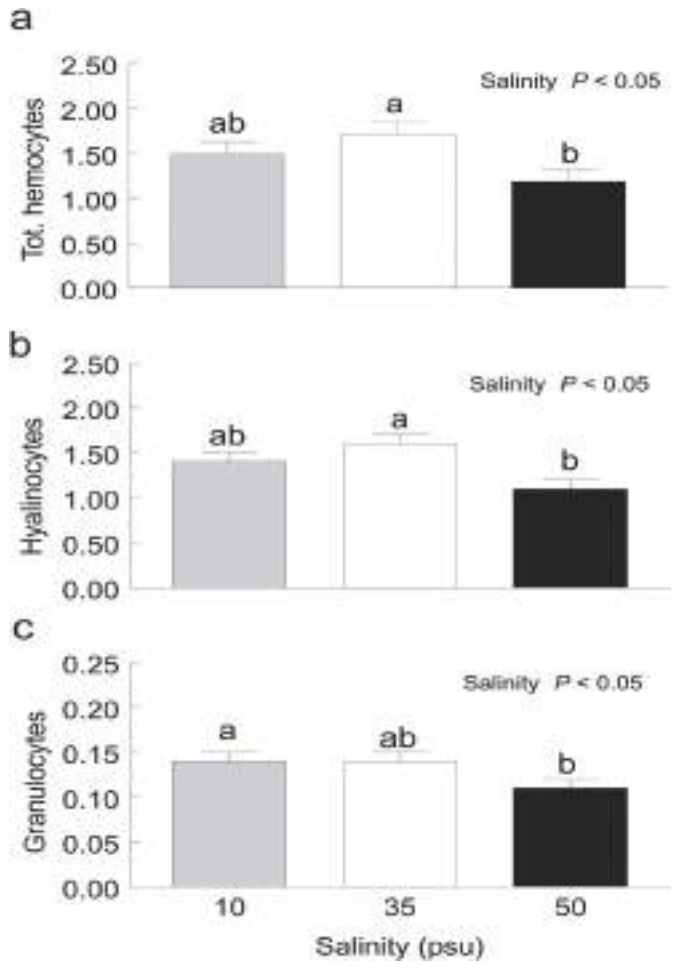

Fig. 5. (a) Total hemocytes, **(b)** hyalinocytes, and **(c)** granulocytes (× 10^6 cells mL^{-1}) in oysters exposed from 1 to 48 h to (HO) hypo–, (C) control and (HP) hyper–osmotic stress conditions (10, 35, and 50 PSU, respectively). Values are global means ± standard error (SE). See Fig. 1 for statistical analyses.

0.05), but no change was observed in exposure time or a significant interaction between salinity and time. The highest glucose content in plasma was observed in oysters exposed to HP stress condition (2.68 ± 0.26 mg dL^{-1}) while the lowest value was obtained in those exposed to HO stress condition (1.60 ± 0.27 mg dL^{-1}) (Fig. 4c).

3.5. Immune responses

3.5.1. Total and differential hemocytes counts

Total hemocyte count (THC) was significantly ($P < 0.01$) different among salinities, and no significant difference occurred in regard to exposure time or interaction between salinity and time. The highest THC was obtained in oysters maintained in C (1.72 ± 0.13 × 10^6 cell mL^{-1}) and HO stress conditions (1.50 ± 0.13 × 10^6 cell mL^{-1}), while the lowest value (1.19 ± 0.13 × 10^6 cell mL^{-1}) was obtained in those exposed to HP stress condition (Fig. 5a).

Similarly, significant differences ($P < 0.01$) were observed in hyalinocyte count among salinities, but no significant effect occurred with exposure time or interaction between salinity and time. Oysters maintained in C and HO stress conditions showed the highest average in hyalinocyte concentration (1.58 ± 0.13 and 1.36 ± 0.13 × 10^6 cells mL^{-1}, respectively), while a lower count of these cells was observed in those exposed to HP stress condition (1.07 × 10^6 ± 0.13 cells mL^{-1}) (Fig. 5b).

Granulocyte cells changed significantly among salinities ($P < 0.05$), but no differences occurred in regard to exposure time or interaction between salinity and time (Fig. 5c). Higher values were obtained in oysters exposed to HO and C conditions (0.142 ± 0.01 and 0.139 ± 0.01 × 10^6 cells mL^{-1}, respectively), while the lowest (0.112 ± 0.01 × 10^6 cells mL^{-1}) was in those exposed to HP stress condition.

3.5.2. Hemocyte viability, phagocytosis and superoxide anion

Significant differences ($P < 0.05$) were obtained in hemocyte viability (%) in relation to salinity condition. The highest values were obtained at HO (99.4 ± 0.4%) and the lowest at HP stress conditions (96.9 ± 0.6%) (Fig. 6a).

No significant differences were found in hemocyte phagocytosis activity in regard to salinity changes. Phagocytosis percentage was observed as follows according to HO (28 ± 12%), C (30 ± 11%), and HP (24 ± 10%) stress condition (Fig. 6b).

In contrast, SOA production was significantly different in relation to salinity changes with higher values at HO and HP stress conditions (1.65 ± 0.19 and 1.56 ± 0.33 arbitrary units of activated/basal hemocytes), while the lowest was obtained at 35 PSU or C condition (0.89 ± 0.11 arbitrary units of activated/basal hemocytes) (Fig. 6c).

4. Discussion

Both salinity induced–stress conditions, particularly salinity increase, exerted a significant effect on the physiological, metabolic and

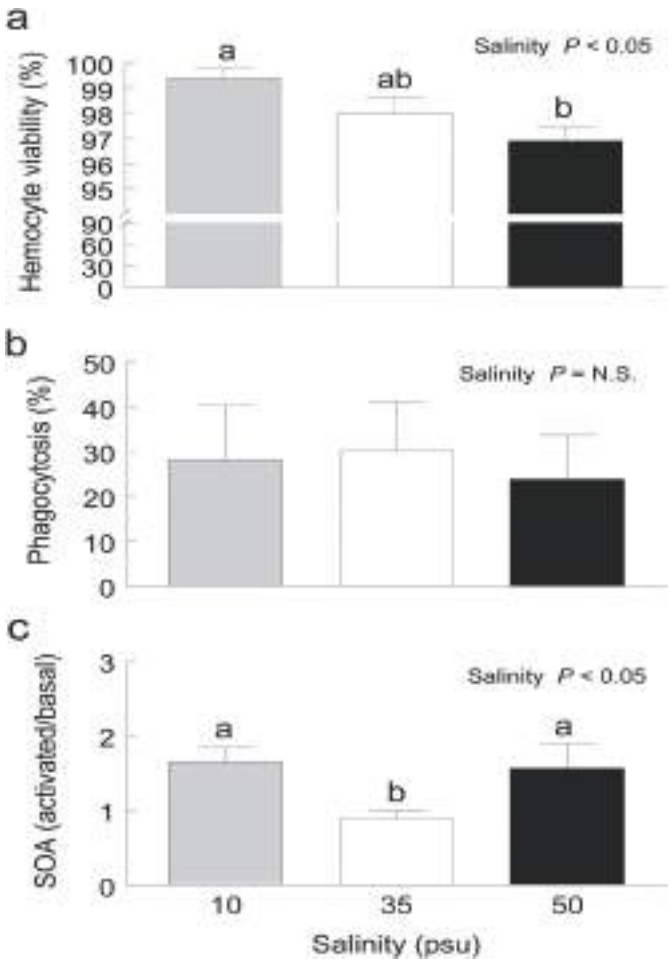

Fig. 6. (a) Hemocyte viability (%), **(b)** phagocytosis (%), and **(c)** superoxide anion (arbitrary units in activated/basal hemocytes) production in oysters exposed 48 h to (HO) hypo–, (C) control, and (HP) hyper–osmotic stress conditions (10, 35, and 50 PSU respectively). Values are means ± standard error (SE). See Fig. 1 for statistical analyses.

immunological responses in *C. corteziensis*. The results demonstrated that this oyster species is less resilient to salinity increase than a decrease, which caused a more stressful condition that could not only increase oyster vulnerability to other environmental stressors but also to opportunistic pathogenic microorganisms as further discussed.

Bivalve mollusks are osmoconformers, which means they lack the ability of maintaining hemolymph osmolality independently of external salinity changes. Instead, osmolality changed concomitantly as environmental osmotic pressure changed when bivalves were induced to a salinity stress condition [10]. However, hemolymph osmolality of *C. corteziensis* reached rearing water osmolality (isosmotic point) at 48 h and 6 h after organisms were induced to HO and HP stress conditions (Fig. 1). In contrast, the isosmotic point of hemolymph with rearing water was reached after 8 h in oyster *C. gigas* exposed to both HO and HP stress conditions [30]. Less time was required to reach the isosmotic point by the mussel *Mitylus edulis* 24 h exposed to HO and 3 h to HP stress conditions [37]. These differences are probably species–specific, which could have been related to the salinity changes that occurred at each geographical zone where the wild populations are located. Thus, *C. corteziensis* is more frequently exposed to HO than HP stress condition at the wild. This result was reflected in the difference of the time it takes to reach the isosmotic point where osmolality was maintained for a higher period of time in oysters exposed to low salinity compared to those exposed to a higher one. Is worth to mention that salinities assayed in this work were chosen similar than used in a previous work, where oyster's growth may still occur at 50 PSU, while at brackish water (20 PSU) occurred the better growth performance [9]. Also, it should be noted that wild oysters experience diurnal and seasonal salinity variations with less extent in both periodicity (*i.e.* depending of tides and sea currents) and intensity (*i.e.* 28.5 PSU for spring and 34 PSU for winter), which significantly differ from those assayed in this experimental trial. However, the results obtained in this research allow determining the physiological, metabolic, and immunological responses of this oyster species when exposed to acute extreme salinity changes, which may occur in the future considering those currently recorded [28]. As observed, osmolality in oyster hemolymph did not change immediately (Fig. 1) despite a notch was made in the oysters' shells to avoid the natural valve closure and seal as a response to salinity stress condition [30,38]. Timing (48 h) was quite remarkable that oysters maintained their osmolality during salinity decrease until they reached that of rearing water. Although the isosmotic point was more rapidly reached (6 h) during salinity increase, 48 h was established as the best sampling time to assess salinity impact on immune response.

Water content in the oyster tissues analyzed showed that salinity induced–stress conditions changed permeability of the cellular membranes, mainly in the mantle (Fig. 2). Interestingly, no differences in gill permeability were observed despite their importance in both function (*i.e.* feeding/respiration) and location in the oyster's body since the gills are easily exposed to the salinity changes that occur in rearing water. The mantle showed the highest variations, particularly in those exposed to HP conditions, which was probably due to the size of the tissue that envelops the oyster's body completely. As expected, hemolymph osmolality was hypo–osmotic with respect to that of rearing water when salinity increased, causing dehydration in the oyster's mantle during the first hour. Then, in the following hour, it was surely compensated by seawater intake which increased water content in the mantle. Finally, at three and 6 h of exposure, water content obtained by intake was discarded with urine, while salt content obtained with seawater was probably used as inorganic osmolytes to increase hemolymph osmolality close to that of rearing water (*i.e.* isosmotic point, Fig. 1). Both inorganic and organic osmolytes regulate osmotic balance, cell volume and function, but organic osmolytes are more compatible with cell physiology [10,39]. Bivalve mollusks use free amino acids as the most commonly organic osmolytes [30,40–44], which decrease when organisms are induced to HO stress condition while inorganic osmolytes remain at constant levels, avoiding cell swelling [44]. In contrast, during HP stress condition, the content of intracellular free amino acids increase through hydrolysis of some proteins [40,45], which avoid cell shrinkage [45,46]. In this research, protein content in the tissues analyzed did not change in salinity induced–stress conditions (Fig. 3). This result suggests that the amino acids contained in protein tissues were not involved as organic osmolytes for regulating osmotic balance and cellular function during the acute salinity induced–stress, which could have been due to exposure time since amino acids are used as organic osmolytes during chronic (≥72 h) salinity induced–stress conditions [30,40,41]. Additionally, some specific peptides and amino acids that are not commonly found as part of structural and functional proteins –rich in particular amino acids as taurine and/or betaine– are used as a source of organic osmolytes as reported in the oyster *Crassostrea gigas* [30,41] and in the mussel *Mytilus edulis* [10]. In contrast to the gills and adductor muscle, the mantle showed an increase –but not statistically significant– in protein content (Fig. 3c), which was probably used as a source of amino acids and organic osmolytes to compensate hemolymph osmolality produced by salinity increase (Fig. 1). The observed changes in protein content in oyster tissues with exposure time to salinity induced–stress conditions seems to be related with the starving condition endured by them during the experimental trial. In the gills and adductor muscle, protein content decreased at the beginning (1–6 h) and increased at the end of the experimental trial (12–48 h). In the mantle, protein content was maintained stable in both oysters induced to HO and C conditions, except for those induced to HP stress conditions, as discussed above.

These results suggest that the mantle –neither gills nor adductor muscle– was firstly involved in osmolality adjustment and cell membrane permeability in *C. corteziensis* induced to HP stress conditions. In contrast, the gills and mantle are considered the main tissues involved in cell adjustment through protein catabolism in *C. gigas* exposed to HO stress conditions [42].

Protein content in *C. corteziensis* plasma was not affected by salinity stress–induced conditions, reflecting that observed in protein content in the tissues analyzed. As discussed above, this result was probably due to oysters exposed to an acute instead of chronic stress condition, where the involvement of protein metabolism and use of specific amino acids have been widely discussed in several bivalve mollusks [10,30,40–44]. Although no significant differences were observed with exposure time, total protein content increased in plasma of those exposed to both HO (0.58 ± 0.08 to 0.77 ± 0.08 mg mL^{-1}) and HP (0.65 ± 0.08 to 0.72 ± 0.08 mg mL^{-1}) stress conditions (data not shown). To avoid cell swelling caused by the increase in membrane permeability in the oyster's tissues, both inorganic and organic osmolytes mostly decreased from in– to outside of the cells and spilled out to the circulatory system, allowing the adjustment of cellular volume and hemolymph osmolality (Fig. 1). The proteins found in the circulatory system could have been involved in respiratory activity and immune response. However, they could also have been used for gluconeogenesis to face salinity induced–stress conditions. In a previous work, the authors observed that oxygen consumption was always higher in *C. corteziensis* exposed to lower– rather than higher salinity, whose physiological condition increased concomitantly as temperature increased [9]. The rate in oxygen demand during salinity stress–induced conditions could explain the highest content of hemocyanin in oysters exposed to HO than those exposed to HP stress conditions (Fig. 4b). Additionally, the protein levels observed in plasma of oysters exposed to HO stress conditions might have been used for hemocyanin synthesis as have been reported in the crab *Carcinus means* [47] and in abalone *Haliotis discus* [48]. Although less likely compared to oxygen consumption rate, it cannot be ruled out that hemocyanin level and total proteins in plasma could have been degraded to amino acids to regulate cell volume in *C. corteziensis* tissues exposed to HP stress condition, as shown for different species of aquatic invertebrates [49,50].

This species metabolism is mainly sustained by carbohydrates (*i.e.* glycogen), with less extent on lipids and proteins, which is more evident during their reproduction cycle [51]. Carbohydrates mainly accumulate in the connective vesicular tissue that envelops all visceral organs and extends them to the mantle [52]. Therefore, glucose is mainly used as a source of energy for different physiological aspects, including stress–induced conditions. This fact has been observed in some osmoregulator species as fishes [53,54] and crustaceans [55,56] induced to salinity stress–conditions. In osmoconformer species the same situation seems to occur, since glycogen content decreased in all tissues of *R. philippinarum* and *R. decussatus* exposed to HO and HP stress conditions [12]. In this study, glucose content in plasma was higher in oysters exposed to HP than HO stress condition (Fig. 4c). Therefore, likely physiological stress and energy requirements increased in high salinity (50 psμ) conditions. This energy must be obtained from carbohydrate reserves (*i.e.* glycogen) accumulated in the oyster's tissues previously mentioned. However, it could also have been obtained through gluconeogenesis from broken–down hemocyanin and total proteins found in the oyster's plasma, as demonstrated in some crustacean and mollusk species [40,45,50]. Moreover, mobilization and use of energy reserves with salinity induced–stress conditions could be explained by a better adaptation of oysters to salinity decrease than increase. The former condition occurs more frequently in their natural habitat (*i.e.* estuaries), as previously discussed.

Immune response has been evaluated in several bivalve mollusks exposed to both acute (*i.e.* $\leq$ 72 h) and chronic (*i.e.* $\geq$ 72 h to four months) salinity stress–induced conditions. Most of the published works have described the effect of salinity decrease rather than increase on the immune response. In general, immune suppression has been observed in some bivalve species exposed to HO stress condition with a particular decrease in counts of total and differential hemocytes, hemocyte viability, hydrolytic enzyme activity, phagocytosis, and reactive oxygen species (ROS) production [18–25]. In contrast, HP stress conditions exerted both stimulation [12,26] and suppression [16,21,23,27] of the immune response in some bivalve species. In this study, salinity exerted changes in the immune response of *C. corteziensis* with quite important differences between HO and HP induced–stress conditions. According to the results, total and differential (*i.e.* hyalinocytes and granulocytes) circulating hemocytes and their viability decreased particularly in oysters exposed to HP stress condition, while no effect was observed in those exposed to HO condition (Fig. 5). The lack of effect on hemocyte counts and their viability with salinity decrease, as observed with some other bivalve species, could have been due to different factors, such as exposure time (acute vs. chronic), change magnitude in terms of salinity units, inter–specific species variations or resilience, physiological condition of organisms, etc. Another possibility is that in most of the previous works, making a notch on bivalve mollusk shell was not considered, as well as the time it takes the organisms to adjust hemolymph osmolality to that of rearing water. It was probably assumed that as osmoconformers this situation occurs instantly. However, it is more likely to occur at different times in a species–specific manner with important differences between HO or HP stress conditions, as demonstrated in this study.

Additionally, with the morphometrical characteristics of *C. corteziensis* hemocytes, their function demonstrated that hemocyte viability significantly decreased as salinity increased (Fig. 6a) while both HO and HP stress conditions increased their oxidative capacity; no significant effect was observed in hemocyte capacity to perform phagocytosis (Fig. 6b and c). These results demonstrated a significant capacity of *C. corteziensis* to deal with salinity decrease but not resist to salinity increase, which caused a suppression of the immune system, as previously mentioned for some other bivalve species. Probably, this oyster species is more adapted to salinity decrease than increase, which could be a particular characteristic shared with some other oyster species. For instance, several physiological mechanisms, particularly shock proteins and apoptosis inhibitors have been identified in *C. gigas* exposed to HO salinity stress condition [57]. So far it is unknown whether these physiological mechanisms also occur when bivalve mollusks are exposed to HP stress condition, which currently occurs with greater frequency and can considerably affect their physiological condition and immune response, as demonstrated in this study.

Salinity conditions could exert even more adverse effects in aquatic organisms, particularly when salinity interacts with some other abiotic factors that could increase oxidative stress with both salinity decrease but even higher with increase, as demonstrated in some invertebrate aquatic species [for a review see Ref. [58]. Temperature exerted an important effect in counts of total circulating hemocytes and phagocytic capacity, but the interaction between salinity and temperature, and salinity by itself has affected only hemocyte phagocytosis in the clam *R. philippinarum* [23]. Recently, a research demonstrated that acidity increase (a decrease in pH) coupled with a decrease in rearing water salinity, decreased the immune response in the mussel *Mytilus coruscus* [20]. As it has occurred with environmental factors, salinity interaction with biotic factors has also exerted a significant effect on the immune response. For instance, an increase in immune response was observed in the clam *R. philippinarum* exposed to hypersaline stress conditions and challenged with *Vibrio tapetis*, the microorganism that caused the brown ring disease [22]. The immune response and infection of the pathogenic bacteria *Listonella anguilarum* was maintained under HO stress condition, but both immune response and disease increased concomitantly as salinity increased, causing immune stimulation by increasing the number of granulocytes in *O. edulis* acclimated to high salinity [27]. A better physiological condition was determined in *C. gigas* acclimated to HO than HP stress conditions, where higher antioxidant capacity and

energetic metabolism have demonstrated they effectively regulate both susceptibility to infection by the ostreid herpesvirus (OsHV–1) and survival of the oysters [59].

Finally, the effects observed in this work on the physiology and immune response in the oyster *C. corteziensis*, together with those observed in relation to feeding and growth rates [9] of this species, suggest the need to determine the lethal limits in relation to salinity changes, in which at least has observed a decrease in heart rate in some species of bivalve mollusks subjected to both hypo and hyper salinity–stress conditions [60]. Also the assessment of lethal limits will permit recognize the optimal conditions, in which the physiology of the oyster is not or less affected (pejus range) by changes in salinity, but also will permit identify the extreme salinity conditions at the oysters could persist in a time–limited manner (pessimum range) [61].

In conclusion, the physiological, metabolic, and immune responses analyzed in *C. corteziensis* showed that this species tolerates acute HO stress conditions, which contrasted with that observed in those exposed to HP where higher energetic metabolism activity (*i.e.* glucose and hemocyanin) and immune system suppression (*i.e.* decrease of both total and differential circulating hemocytes and cell viability) seems to compromise the physiological condition of the oysters. Additionally, the metabolic responses showed higher stressful conditions in oysters exposed to HP than HO stress conditions. This result is more important considering the actual salinity changes worldwide, where particularly salinity increase could impair different physiological aspects of this species. Furthermore, it is even more relevant with the interaction of some other environmental factors (*i.e.* increase of both temperature and/or acidity of the oceans) and the increase in susceptibility of oysters to different pathogenic microorganisms, which certainly cause different diseases that mine both wild population and also farmed oysters, causing important economic losses.

Declaration of competing interest

As authors we declare that we do not known competing financial interests or personal relationships that could have appeared to influence the work reported in this manuscript.

CRediT authorship contribution statement

Ricardo Pérez–Velasco: Conceptualization, Investigation, Formal analysis, Writing – review & editing. **Marlenne Manzano–Sarabia:** Investigation, data interpretation, Writing – review & editing. **Miguel Ángel Hurtado–Oliva:** Conceptualization, Funding acquisition, Project administration, Investigation, Formal analysis, Writing – original draft.

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